

AD _____
(Leave blank)

Award Number:
W81XWH-07-1-0628

TITLE:
Identification of Substances for Ubiquitin-Dependent Proteolysis During Breast Tumor Progression

PRINCIPAL INVESTIGATOR:
Charles H Spruck, Ph.D.

CONTRACTING ORGANIZATION:
**Sidney Kimmel Cancer Center
10905 Road to the Cure
San Diego, CA 92121**

REPORT DATE:
October 2008

TYPE OF REPORT:
Final

PREPARED FOR: **U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012**

DISTRIBUTION STATEMENT: (Check one)

- ☒ Approved for public release; distribution unlimited
- ☐ Distribution limited to U.S. Government agencies only;
report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 14-10-2008		2. REPORT TYPE Final		3. DATES COVERED (From - To) 15 SEP 2007 - 14 SEP 2008	
4. TITLE AND SUBTITLE Identification of Substances for Ubiquitin-Dependent Proteolysis During Breast Tumor Progression				5a. CONTRACT NUMBER W81XWH-07-1-0628	
				5b. GRANT NUMBER W81XWH-07-1-0628	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Charles H. Spruck, Ph.D.				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Sidney Kimmel Cancer Center 10905 Road to the Cure San Diego, CA 92121				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Ubiquitylation is post-translational modification in which a small and highly abundant protein called ubiquitin is attached to proteins. Ubiquitylation regulates several processes that are central to breast tumorigenesis, including cell division, inflammation, and angiogenesis. However, defining how ubiquitylation contributes to breast tumorigenesis has been technically limited. We developed an innovative methodology that utilizes protein microarrays as a platform to evaluate the ubiquitylation activity of breast tumor specimens on a proteome-wide scale. In this proposal, we utilized this methodology to define changes in ubiquitylation activity during breast tumor progression. Extracts from breast tumors of either low or high grade/stage were profiled and ubiquitylation activity (fluorescence intensity) quantified for >8,000 substrates on the protein microarray. Several distinct differences in ubiquitylation activity (>2-fold) were observed, with many of the substrates being involved in processes such as cell division, angiogenesis, and metastasis. Several targets of ubiquitylation were then validated. The results of this study show that distinct changes in ubiquitylation activity accompany the progression of breast tumors to more advanced disease. These activities likely drive breast tumor progression and could potentially represent novel targets for therapeutic intervention.					
15. SUBJECT TERMS ubiquitin, protein microarrays, breast tumor progression					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 31	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusion.....	8
References.....	8
Appendices.....	8
- Manuscript attached (23 pages)	

A. Introduction

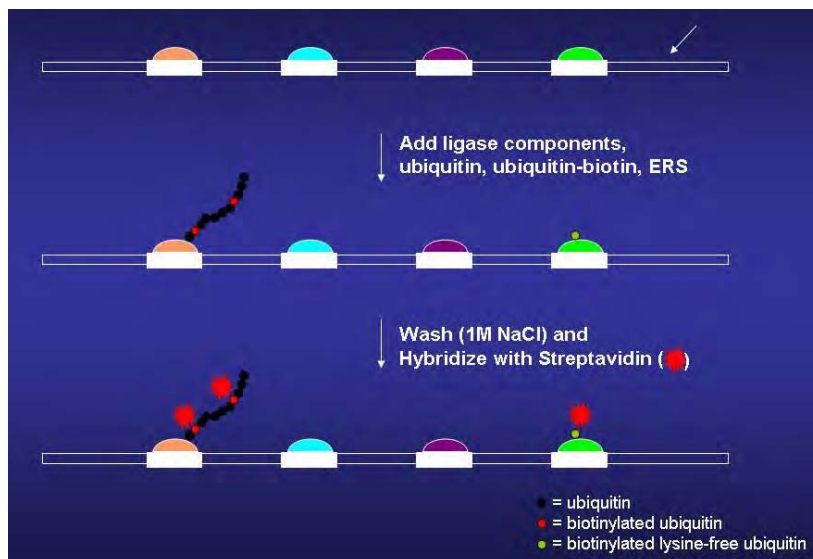
Ubiquitylation is an enzymatic process of protein modification present in all cells in which a small and highly abundant protein called ubiquitin is physically attached to other proteins. The attachment of ubiquitin to proteins can have several consequences, including activating its function or targeting it for destruction. Ubiquitylation is an important regulatory process in cellular division. However, recent experimental evidence has shown that ubiquitination activity is altered in breast cancers and contributes directly to tumorigenesis.

Understanding how ubiquitination contributes to breast tumorigenesis has been problematic because no experimental tools existed that can identify which proteins are ubiquitylated in cells. To overcome this limitation, we have developed an innovative experimental assay that enables the identification of ubiquitylated proteins using protein microarrays, containing >8000 human proteins dotted onto glass slides. We have successfully used our assay to identify substrates of purified ubiquitylating enzymes and complex biological mixtures such as breast tumor extracts. The goal of this study is to identify changes in ubiquitylation activity associated with the transformation of normal breast epithelial cells into breast cancers.

B. Body

Establishment of a protein microarray-based ubiquitylation activity profiling assay. Our methodology for profiling ubiquitylation activity in breast tumor specimens is based on protein microarrays, glass microscope slides spotted with >8,000 human recombinant proteins, which serve as substrate for *in vitro* reactions (Fig. 1). Ubiquitylation activity is profiled by performing “on-chip” ubiquitylation reactions that include extract from a breast tumor specimen and biotin-labeled ubiquitin. The substrates of ubiquitin conjugation are then detected by incubation of the protein microarrays with fluorescein-labeled streptavidin and visualized using a fluorescence slide reader.

Figure 1. Overview of “on-chip” ubiquitylation reactions using protein microarrays as substrate. Protein microarrays containing >8,300 human recombinant proteins spotted onto glass microscope slides are used as substrates for *in vitro* ubiquitylation reactions containing biotin-labeled ubiquitin. Substrates are then detected using fluorescein-labeled streptavidin. We have successfully employed this methodology to profile the ubiquitin ligase activity of purified ubiquitin ligases and extracts prepared from human and mouse cell lines and primary tumors.



We first focused our efforts on refining our assay system to achieve maximal activity on the protein microarrays. We took this approach since the protein microarrays are expensive

(\$1,500/each) and believed that the assay should be well-defined before utilizing these materials. In collaboration with Invitrogen Corp., we first created custom protein microarrays which are spotted with ~20 different human recombinant proteins that we showed were ubiquitylated by the extracts of dividing cells. These custom arrays were created by a protein spotting machine at the Invitrogen facility. Using these custom protein microarrays, we established optimal conditions for profiling ubiquitylation activity for the breast tumor extracts (data not shown). Using these optimal conditions, we then performed test reactions on the full protein microarrays (Fig. 2). We tested extracts prepared from rabbit reticulocytes and S100 fractions of HeLa cells, both known to contain robust ubiquitin ligase activity. The results of these experiments showed that these extracts were capable of ubiquitylating >400 substrates on the protein microarray, including several substrates known to be regulated by the ubiquitin-proteasome system (UPS), as well as many novel substrates (Fig. 2).

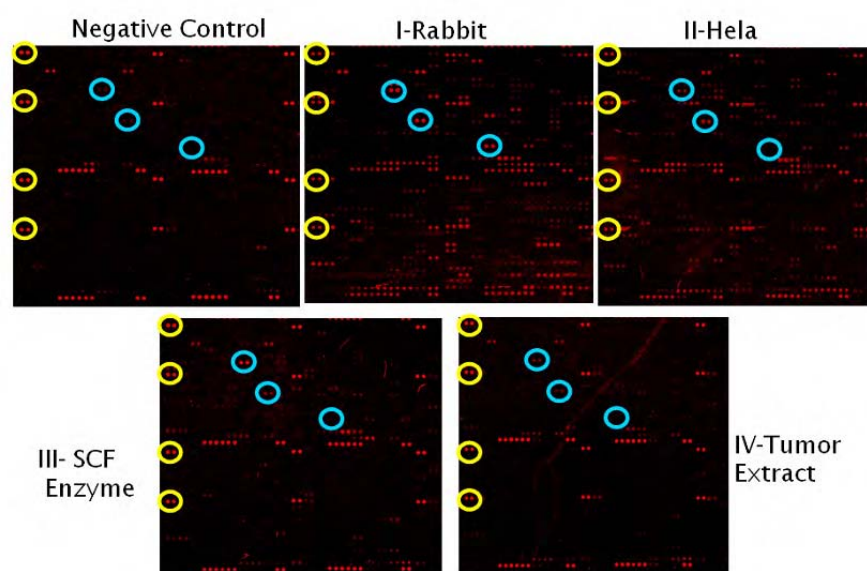


Figure 2. Protein microarray-based analysis of ubiquitylation activity in cell and tumor extracts. Ubiquitylation reactions were performed using extract prepared from rabbit reticulocytes (upper middle) and HeLa cells (upper right), whole breast tumor extract (lower right), and purified recombinant SCF^{Skp2} (lower left). Negative control includes E1, E2, ATP regeneration system, ubiquitin, and biotin-ubiquitin. Positive controls for fluorescence (yellow circles) and examples of ubiquitylated substrates (blue circles) are shown.

Profiling ubiquitylation activity of breast tumor specimens. We next obtained fresh-frozen breast tumor specimens that were either of low or high pathological stage and grade from the Tumor Bank at the Sidney Kimmel Cancer Center, SKCC). Extracts were generated in ubiquitylation reaction buffer (20 mM Hepes (pH 7.4), 2.5 mM MgCl₂, 0.5 mM DTT) and protein concentration determined. We then performed ubiquitylation reactions by overlaying tumor extract (50 µg), E1 activating enzyme, ubiquitin, biotin-labeled ubiquitin, and an ATP regeneration system on the full protein microarrays (ProtoArrays, Invitrogen). Reaction mixtures (100 µl) were cover-slipped, and the slides incubated @ 37°C for 1hr in a humidified chamber. Reactions with no ATP regeneration system served as controls. Following a wash in 1M NaCl for 30 min to remove non-covalent interactions, the slides were incubated with fluorescein-labeled streptavidin for 1hr @ 25 °C with shaking. The slides were then analyzed using a Genepix 400B Scanner and fluorescence intensity of each spot quantified with Genepix

Pro 6.0 software. The data was then imported in Microsoft Excel and analyzed. Based on control reactions, we chose 500 fluorescence counts as an arbitrary cut off for ubiquitylation activity. We then analyzed the data sets for differences in ubiquitylation between the low and high grade/stage breast cancers. We chose a >2-fold difference in fluorescence intensity at any particular spot as a cut off for a difference between groups. Using these parameters, we found several differences in ubiquitylation activity between low and high grade/stage breast tumors (Table 1). We then performed a literature search of the differentially ubiquitylated substrates and found that many have defined roles in processes such as DNA repair, cell adhesion, apoptosis, and tumor invasion.

Table 1. Ubiquitylation changes associated with breast tumor progression

UP	FUNCTION	DOWN	FUNCTION
RAD23A	Involved in post-replication repair of UV-damaged DNA	FGFR2	Metastasis, EMT
TANK	TRAF-associated NF-kappaB activator	FLT3	RTK, AML
FES/FPS	protein tyrosine kinase	SULF1	Inhibits tumor growth
ZNF364	BCA E3 ligase	AIM2	Induces apoptosis
ABL1	Proto-oncogene, cell adhesion, cell division	PRRG1	SH3 recently shown to bind ubiquitin
ZAP70	TCR signaling and development	PLK3	Mitosis, DNA damage checkpoint activation
PELI1	Adaptor protein of IRAK1	UCHL3	DUB
WEE1	Cell cycle kinase	BC001294	unknown
TFPI2	tissue factor pathway inhibitor2		
BC050434	unknown		

Low vs. High Grade Tumors

Validation of differentially ubiquitylated substrates. We next determined the efficacy of our assay in detecting substrates of ubiquitin conjugation activity, we randomly selected 50 substrates identified on the protein microarrays as ubiquitylated and performed a literature search for evidence for their regulation by ubiquitylation. In 34 cases, the proteins were found to be either regulated by ubiquitin-dependent proteolysis or were known binders of ubiquitin. This represented a nearly 20-fold enrichment over a randomly selected proteins displayed on the protein microarray. We next selected several substrates identified as ubiquitylated on the protein microarrays but not reported in the literature and attempted to validate their ubiquitylation *in vivo*. We first cloned the cDNAs for the putative ubiquitylated substrates (obtained as Image clones from Open Biosystems) into the expression vector pFlag-CMV2 (Sigma). The substrate expression plasmids were then co-transfected with a HA-ubiquitin expression plasmid into HEK293T cells. After 48 hrs, the cells were incubated in media containing 10 μ M of proteasome inhibitor MG132 for 4-6 hrs to stabilize ubiquitylated intermediates. The cells were then lysed in 1% SDS to dissociate protein complexes (denaturing immunoprecipitation), the buffer reconstituted to 1x RIPA buffer and extracts immunoprecipitated with anti-Flag antibodies (Sigma). The immunoprecipitates were then separated on SDS-PAGE gels and blotted to nitrocellulose membranes. The membranes were then probed with anti-HA antibodies (Covance) to detect ubiquitylated species. An example of this analysis is shown in Fig. 3. In all cases tested (4/4), the substrate was found to be a target of modification activity *in vivo*.

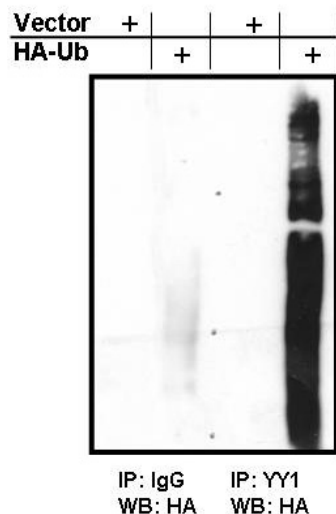


Figure 3. Validation of ubiquitin modifications *in vivo*. Ubiquitylation of YY1. HEK293T cells were transfected with HA-ubiquitin expression plasmids, extracts prepared, and YY1 immunoprecipitated. Western blot analysis using anti HA-antibodies show a smear of poly-ubiquitylated YY1.

Summary of SOW items completed

SOW #	Status	Comments
1	Completed	
2	Completed	
3	Completed	
4	Completed	
5	Completed	
6	Not-completed	Complemented by additional experiments in 8
7	Completed	
8	Completed	<i>In vivo</i> analysis performed
9	Completed	

C. Key research Accomplishments

1. Profiled ubiquitylation activity of primary breast tumor specimens on a proteome-wide scale on protein microarrays.
2. Identified differences in ubiquitylation activity associated with breast tumor progression.
3. Validated substrates that demonstrated differential ubiquitylation activity as ubiquitylated.

D. Reportable Outcomes

1. Methodology of protein ubiquitylation assay using protein microarrays as a substrate platform.
2. Characterization of differences in breast tumor ubiquitylation associated with breast cancer progression.

(See Appendix for submitted manuscript)

This methodology was presented at the 2008 Annual HUPO Meeting in Amsterdam, Netherlands.

E. Conclusions

We have found that key changes in ubiquitylation activity occur as breast tumors progress to advanced disease. The substrates of this activity include several proteins involved in key processes such as DNA repair, cell adhesion, apoptosis, and tumor invasion. The nature of these substrates together with our validation experiments adds confidence that this *in vitro* assay is faithfully identifying substrates of ubiquitylation activity *in vivo*. This assay could be applied to other significant problems in breast cancer, such as identify post-translational activity associated with breast cancer metastasis, the chemotherapeutic response, or associated with specific properties of the breast cancer.

G. References

None

H. Personnel Paid from Grant

Charles H. Spruck, Ph.D., Principle Investigator
Dahui Sun, Senior Research Associate

I. Appendices

Manuscript, Profiling Post-translational Modification Activity of Complex Biological Mixtures Using Protein Microarrays, by Sonia V. del Rincón, Jeff Rogers, and Charles Spruck.

J. Supporting Data

None

Profiling Post-translational Modification Activity of Complex Biological Mixtures Using Protein Microarrays

Sonia V. del Rincón^{1,*}, Jeff Rogers^{2,*}, and Charles Spruck^{1,3}

¹Sidney Kimmel Cancer Center

10905 Road to the Cure

San Diego, California 92121, USA

²Invitrogen Corporation

1600 Faraday Avenue, Carlsbad

California 92008, USA

*These authors contributed equally to this manuscript

³Corresponding author: Charles Spruck, Sidney Kimmel Cancer Center, 10905 Road to the Cure, San Diego, California 92121, USA. Tel: 858-450-5990; Fax: 858-450-3251; email: cspruck@skcc.org

Running Title: Post-translational modification profiling on protein microarrays

Abbreviations: PTM, post-translational modification; Ubl, ubiquitin-like; SUMO1, small ubiquitin-like modifier 1; NEDD8, neural precursor cell expressed and developmentally down-regulated 8.

Summary

Post-translational modifications (PTMs) can alter the expression, location, and function of proteins thereby achieving a greater functional diversity of the proteome. However, the evaluation of PTMs on a proteome-wide scale has proven technically difficult. To facilitate these analyses we have developed an assay system that uses protein microarrays as a platform to profile PTM activity in complex biological mixtures such as cellular extracts or pathological specimens. Reactions are performed 'on-chip' and modified substrates are identified via a fluorescent signal. Here, we apply this methodology to three molecularly complex PTMs; ubiquitylation, SUMOylation, and NEDDylation. We further validate this methodology by confirming the *in vivo* modification of several novel PTM substrates. This technique offers several advantages over currently used PTM detection methods, including ease of use, rapidity, scale, sample source diversity, and the ability to multiplex analyses. Furthermore, by allowing for the intrinsic enzymatic activities of cell populations or pathological states to be directly compared, this methodology has wide-spread applications for the study of PTMs in human diseases.

Introduction

Post-translational modifications (PTMs) are essential for the proper function of many proteins and dysregulation of this process plays a causative role in several human diseases (reviewed in (1)). Modifications ranging from the simple conjugation of a phosphate group to the complex addition of ubiquitin can drastically alter the function of a protein. For example, the conjugation of ubiquitin to a substrate can modulate its activity, target it for degradation, alter its cellular location, or determine its interaction with other proteins (2). However, despite the importance of these modifications in maintaining cellular homeostasis and promoting human diseases, identifying which proteins are modified by PTMs in mammalian cells on a proteome-wide scale has proven technically difficult.

To overcome these technical limitations, we explored the possibility of using protein microarrays as a platform to profile PTM activity. To date, the analysis of PTMs using protein microarrays has been limited to the phospho-proteome and profiling substrates of purified yeast enzymes (3-5). Phosphorylation is a 'simple' PTM compared to the complex enzymatic cascades required for many other modifications such as the conjugation of ubiquitin and ubiquitin-like (Ubl) proteins (e.g. SUMO1 and NEDD8). These modifications are mediated by multi-step enzymatic reactions involving an activating (E1), conjugating (E2), and ligase (E3) enzyme, that selectively transfers the PTM to substrates (1).

Experimental procedures

Extract Preparation. Cell (HeLa, mouse and human fibroblasts) and tumor (human breast) specimens were suspended in 20 mM Hepes (pH 7.4), 2.5 mM MgCl₂, 0.5 mM DTT, 5 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 2 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin, and the cells disrupted by brief sonication. The extracts were clarified by centrifugation for 15 min at 14,000 x *g* and then snap-frozen in liquid nitrogen until use. Rabbit reticulocyte lysate and HeLa S-100 fractions were purchased from Boston Biochem.

Recombinant Proteins. Human SCF^{Skp2} complexes were produced in Sf9 cells as described previously (6). Recombinant Cks1 was produced in bacteria and purified as described (7). Cyclin A-Cdk2 complexes were purchased from Invitrogen.

Antibodies. Antibodies used in substrate detection on protein microarrays included: anti-ubiquitin (Biomol, PW8805); anti-SUMO1 (Zymed, 33-2400); anti-NEDD8 (Zymed, 34-1400); and anti-phosphate (Zymed, 61-8300). For multiplex experiments, antibodies were labeled with Alexa Fluor NHS ester dyes AF488, AF532, AF594, and AF647 according to the manufacturer (Invitrogen). Antibodies used in substrate validation experiments included: anti-YY1 (Santa Cruz Biotechnology); anti-IGF-1R (Zymed); anti-HA (Covance); anti-Flag (Sigma); and anti-Myc (9E10, Santa Cruz Biotechnology).

PTM Profiling. Extracts (2-400 μg in a volume of 40 μl suspension buffer) were combined with 5 μM MG-132 and 4 μM of the relevant aldehyde (ubiquitin, SUMO1, or NEDD8; Boston Biochem), and incubated at 25 °C for 15 min. The reactions were then supplemented with modifier (1.25 $\mu\text{g/ml}$), biotin-labeled modifier (50 ng/ml), Tween-20 (0.1%), energy regenerating system (Boston Biochem), and 1 x reaction buffer (ubiquitylation, SUMOylation, NEDDylation buffers, Boston Biochem) in a 100 μl final volume. When antibodies were used for PTM detection, biotin-labeled modifiers were omitted from the reaction. For multiplex analysis of ubiquitin, SUMO1, NEDD8, and phosphorylation, 1 x ubiquitylation buffer was used. For SCF^{Skp2} experiments, reaction conditions were as described (6). The reaction mixtures were applied to Human ProtoArrays (Invitrogen), cover-slipped, and incubated at 37°C for 1 hr in a humidified chamber. The ProtoArrays were then washed for 10 min in PBS-Tween (0.1%, PBST) containing 1M NaCl, 2 x 10 min in PBST, and then incubated in Streptavidin Alexa Fluor 647 (100 ng/ml) for 1 hr at 25°C. For antibody detection experiments, the ProtoArrays were incubated with the relevant Alexa Fluor-labeled antibodies in PBST for 16 hrs at 4°C. The ProtoArrays were then washed 3 x 10 min in PBST and spun dry. Imaging was performed using a GenePix 4000B Slide Imager (Molecular Devices) and spots analyzed using GenePix Pro software. Gal files (which contain array production information—spot location, identification, and quantification) were downloaded from www.invitrogen.com and used with GenePix Pro software to analyze the median intensity of each spot.

Substrate Validation Experiments. HEK293T cells (ATCC) were either transfected with plasmids that express HA-tagged ubiquitin (ubiquitin), co-transfected with Myc-NEDD8 and Flag-PAK3 or Flag-MUSK (NEDD8), or non-transfected (SUMO1). Cells were then lysed in 1% SDS (containing 20 mM N-ethyl-maleimide (NEM)) to disrupt non-covalent interactions, and the buffer adjusted to 0.1% SDS, 0.5% NP40, 20 mM NEM, 50 mM Tris (pH 8.5), 150 mM NaCl, 5 mM EDTA, 5 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 2 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin. For ubiquitin experiments, extracts were immunoprecipitated with anti-YY1 antibodies and ubiquitylation determined by Western blot analysis using anti-HA antibodies. For SUMO1, the extracts were immunoprecipitated with anti-IGF-1R antibodies and Western blots probed with anti-SUMO1 antibodies. For NEDD8, the extracts were immunoprecipitated with anti-Myc antibodies and Western blots probed with anti-Flag antibodies. In all cases, immunoprecipitation of extracts with IgG antibodies of the same species served as control.

Results

A schematic of our methodology is shown in Figure 1a. Reactions are performed ‘on-chip’ by overlaying the protein microarrays with a purified conjugation enzyme or extract prepared from a biological specimen (e.g. cell or pathological specimen) and any required factors. The protein microarrays are spotted with ~8,000 different human recombinant proteins in duplicate which serve as substrates for PTM conjugation. The substrates of PTMs are subsequently “tagged” by conjugation of a labeled-modifier (e.g. biotin-labeled) present in the reaction mixture. Following a stringent wash to remove non-covalent interactions, the conjugated substrates are then detected using ‘binders’ labeled with fluorescent dye (e.g. antibodies or strepavidin) and the fluorescent signals quantified using a standard slide reader.

As proof of principle, we first sought to determine whether complex PTMs could be profiled using protein microarrays as a platform. Using ubiquitylation as a model system, we profiled substrates of the ubiquitin ligase SCF^{Skp2}, which has a well-defined role in human tumorigenesis (5). SCF^{Skp2} is known to ubiquitylate several substrates including the cyclin dependent kinase inhibitor p27^{Kip1} (6, 8). This reaction is molecularly complex and requires: 1) substrate phosphorylation; 2) association of the substrate with cyclin A-Cdk2 complexes; and 3) the co-factor Cks1. We performed ubiquitylation reactions that included recombinant human SCF^{Skp2}, E1 and E2 enzymes, ATP regeneration system, ubiquitin, and biotin-labeled ubiquitin. The results of these experiments showed that p27^{Kip1} could be efficiently ubiquitylated on the protein microarray by SCF^{Skp2}, and conjugation activity was only present when all the required

components were added to the reaction (Fig. 1b). In addition to p27^{Kip1}, several novel substrates of SCF^{Skp2} were also identified (Fig. 1b).

We next tried various perturbations of the ubiquitylation reaction to determine the optimal assay conditions. We evaluated different slide surface chemistries, reaction buffers, assay conditions, and detection methods. PATH slides (glass slides coated with nitrocellulose) proved to be superior to epoxy or hydrogel slides in reducing background (data not shown). The addition of 0.1% Tween-20 to both the reaction and wash buffers also significantly limited background and had little effect on PTM conjugation activity. Furthermore, the addition of inhibitors of de-conjugating enzymes (e.g. ubiquitin-aldehyde) was found to increase PTM conjugation activity (data not shown).

Using optimized conditions, we next sought to determine whether this methodology could be used to profile the PTM activity of complex biological mixtures, such as cellular extracts or pathological specimens. We first tested rabbit reticulocyte lysates and S-100 fractions from human HeLa cells, both known to contain robust ubiquitylation activity. These extracts were found to efficiently ubiquitylate many substrates on the protein microarrays (reticulocytes- 249; S-100 fraction- 119 on the protein microarrays (Fig. 1c, Table 1). Summarized in Table 2 are 66 substrates of ubiquitylation activity detected with either rabbit reticulocyte lysate or HeLa S-100 fractions. Several of these substrates were previously shown to either bind ubiquitin (e.g. LIVIN (9), RNF4 (10), ZNF364 (11)), contain ubiquitin binding domains (e.g. CUED1C (12), RAD23A (13)), or are known substrates of ubiquitylation activity (e.g. FLT1 (14), JAK2 (15), INSR (16)), lending strong support that this methodology can

detect true substrates of ubiquitin conjugation activity. Similarly, whole-cell extracts prepared from various cultured cell lines of both human and mouse origin were also found to efficiently ubiquitylate many (~120) substrates on the protein microarrays (Table 1). Approximately half of these substrates were found to be consistently ubiquitylated by all the cellular extracts profiled. Collectively, these results demonstrate that this methodology could be used to profile the PTM activity of complex biological specimens of various species origin. Of note, the frequency of ubiquitylated substrates detected on the protein microarrays compares favorably to several proteome-wide analyses of ubiquitylation using mass spectrometry as a platform (17, 18).

A clinically relevant application of this methodology is comparative profiling, wherein disease-associated changes in PTM activity are compared, for example, in normal versus diseased state tissues or during disease progression. To this end, we next profiled the ubiquitylation activity of extracts prepared from human pathological breast tumor specimens that had been kept frozen at -80°C for >10 years. Remarkably, these extracts were found to contain robust ubiquitin-conjugation activity (Table 1); comparable to that observed for cellular fractions or whole-cell extracts prepared from cultured cells. Next, we pooled extracts from 5 low-grade and 5 high-grade tumors and applied to the protein microarrays. The results of this analysis showed that differences in ubiquitin conjugation activity occurred as tumors progressed to more advanced stages (Table 3). Interestingly, several of the differentially modified substrates were found to have defined roles in processes that are central to tumor progression (e.g. growth control, DNA repair, angiogenesis).

We next determined whether this methodology could be easily adapted to other complex PTMs, such as SUMO1 (small ubiquitin-like modifier 1) and NEDD8 (neural precursor cell expressed and developmentally down-regulated 8). SUMO1 and NEDD8 are conjugated to substrates in complex multi-step enzymatic cascades similar to but distinct from ubiquitylation (19). Reaction conditions used were similar to those used for ubiquitin (described above) except for the substitution of the relevant reaction buffer, E1 enzyme, aldehyde derivative, and biotin-labeled modifier (see Methods). The results of these experiments showed that HeLa cell extracts were capable of conjugating SUMO1 and NEDD8 to many substrates on the protein microarrays (Table 2). Of the SUMOylated substrates identified, HIPK3 (20) and RNF4 (10) were previously shown to bind SUMO1, and the majority of the remainder of substrates contained consensus SUMO targeting sequences (yKxE/D) (21). Although few substrates of NEDDylation have been identified (22-24), our screen did detect one putative NEDD8 target, LGALS3, that was previously identified using an alternative proteomic approach (24).

We next explored whether multiple PTM activities could be simultaneously analyzed on a single protein microarray. For these multiplex experiments, antibodies specific for ubiquitin, SUMO1, NEDD8, and phosphorylated amino acids were labeled with different fluorescent dyes of non-overlapping excitation and emission wavelengths (see Methods). The protein microarrays were first incubated with HeLa cell extracts supplemented with an ATP regenerating system, the various E1 enzymes, aldehydes, and modifiers, and subsequently probed with a mixture of the four PTM detection antibodies. The results of these experiments showed that multiple PTM activities could

be simultaneously profiled on the same protein microarray, with some substrates conjugated to multiple modifiers (Fig. 1d).

To determine the efficacy of this methodology in profiling PTM activity, we selected several PTM substrates that were identified on the protein microarrays but had not been previously reported in the literature and determined whether they were indeed modified *in vivo*. HEK293T cells were transfected with plasmids that express a tagged-modifier, the putative substrates then immunoprecipitated from the extracts, and PTM conjugation determined by Western blot analysis. YY1, a regulator of the ubiquitin ligase MDM2 that controls the ubiquitin-dependent proteolysis of p53 (25), was found to be poly-ubiquitylated in HEK293T cells (Fig. 2a). Additionally, insulin-like growth factor 1 (IGF-1R), a receptor tyrosine kinase that mediates IGF1 signaling (25), was found to be conjugated to SUMO1 *in vivo* (Fig. 2b). Furthermore, p21^{Cip1}-activated kinase 3 (PAK3), which is associated with non-syndromic mental retardation in humans (26), and MUSK, a receptor tyrosine kinase that plays a role in neuromuscular junction organization (27), were both found to be conjugated to NEDD8 (Fig. 2c). These results confirm that the PTM activity detected on the protein microarrays was indeed present *in vivo*.

Discussion

Currently used techniques for profiling PTM activity on a proteome-wide scale have included two-hybrid and high-copy suppressor screens in yeast, and mass spectrometry (24, 28-30). However, several limitations exist with these techniques. For example, mass spectrometry-based analyses of PTMs is often complicated by: 1) low substrate abundance, a characteristic of many ubiquitylated proteins, and/or a sub-stoichiometric level of the PTM; 2) the labile nature of many PTMs, making their preservation through biochemical purification, separation techniques, fragmentation, and analysis problematic, especially if native conditions are required leaving substrates vulnerable to de-conjugating enzymes; 3) the adverse effects of certain PTMs on proteases, ionization, and detection efficiency; and 4) multi-site or multi-species modifications, which could make data interpretation problematic.

Our methodology overcomes many of these limitations and provides several distinct advantages. Since the assay measures the intrinsic enzymatic activity of a specimen, it is less sensitive to substrate concentration and sub-stoichiometric modifications are easily detected. The reactions can be performed using crude extracts, eliminating elaborate purification protocols that may promote PTM de-conjugation. The methodology can also be easily adapted to the analysis of other PTMs, and multiplexing PTM analyses on the same protein microarray is possible and interpretation of data is not overly complicated. Additionally, the assay can be performed with “natural” modifiers (if antibodies are used for detection) circumventing any potential biases caused by labeled-modifiers. Furthermore, the reactions are

sensitive (can be performed with as little as 2 μ g of extract) and analysis completed in a single day.

However, there are several potential limitations with our assay. Firstly, the latest generation protein microarrays display ~8,000 human proteins, representing only 1/3 of the proteome. Secondly, a portion of the substrates on the protein microarrays could be misfolded. Thirdly, being an *in vitro* assay, various *in vivo* regulations (e.g. temporal or spatial regulations) will likely be lost during extract preparation. Finally, information regarding the type or site of PTM attachment to substrates cannot be ascertained however, this information could possibly be obtained by coupling the analysis with mass spectrometry.

An important potential application of this methodology is the ability to compare PTM activities between different cell populations or pathological states. In combination with genetic mutants, small molecule perturbants, or RNAi technology, this methodology could help to define both substrate-specific and global aspects of PTMs. Considering that dysfunction of PTMs play a critical role in the initiation and progression of a number of human pathological states, this methodology is an important step forward in the field of proteomics because it allows for the identification of aberrant PTM patterns in human diseases using cell extracts, specific organelles, or fresh or archival tissue specimens. Modified cell lines, disease model systems and specialized tissues all lend themselves well to PTM profiling using this methodology, with the ultimate goal of furthering our understanding of disease states and identifying novel therapeutic targets for their treatment.

Acknowledgements

We thank Steven Reed of The Scripps Research Institute (TSRI) for recombinant Cks1 protein. This work was supported by a grant from the Department of Defense Breast Cancer Research Program (DOD BCRP) to C.S. S.V. d R is supported by a postdoctoral multidisciplinary-award from the DOD-BCRP.

References

1. Ciechanover, A. (1998). The ubiquitin-proteasome pathway: on protein death and cell life. *EMBO J* **17**, 7151-7160.
2. Welchman, R. L., Gordon, C., and Mayer, R. J. (2005). Ubiquitin and ubiquitin-like proteins as multifunctional signals. *Nat Rev* **6**, 599-609.
3. Ficarro, S. B., McClelland, M. L., Stukenberg, P. T., Burke, D. J., Ross, M. M., Shabanowitz, J., Hunt, D. F., and White, F. M. (2002). Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat Biotechnol* **20**, 301-305.
4. Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X., Fasolo, J., Guo, H., Jona, G., Breitkreutz, A., Sopko, R., McCartney, R. R., Schmidt, M. C., Rachidi, N., Lee, S. J., Mah, A. S., Meng, L., Stark, M. J., Stern, D. F., De Virgilio, C., Tyers, M., Andrews, B., Gerstein, M., Schweitzer, B., Predki, P. F., and Snyder, M. (2005). Global analysis of protein phosphorylation in yeast. *Nature* **438**, 679-684.
5. Gupta, R., Kus, B., Fladd, C., Wasmuth, J., Tonikian, R., Sidhu, S., Krogan, N. J., Parkinson, J., and Rotin, D. (2007). Ubiquitination screen using protein microarrays for comprehensive identification of Rsp5 substrates in yeast. *Mol Sys Biol* **3**, 1-12.
6. Spruck, C., Strohmaier, H., Watson, M., Smith, A. P., Ryan, A., Krek, T. W., and Reed, S. I. (2001). A CDK-independent function of mammalian Cks1: targeting of SCF(Skp2) to the CDK inhibitor p27Kip1. *Mol Cell* **7**, 639-650.
7. Bourne, Y., Watson, M. H., Hickey, M. J., Holmes, W., Rocque, W., Reed, S. I., and Tainer, J. A. (1996). Crystal structure and mutational analysis of the human CDK2 kinase complex with cell cycle-regulatory protein CksHs1. *Cell* **84**, 863-874.
8. Ganoth, D., Bornstein, G., Ko, T. K., Larsen, B., Tyers, M., Pagano, M., and Hershko, A. (2001). The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27. *Nat Cell Biol* **3**, 321-324.
9. Ma, L., Huang, Y., Song, Z., Feng, S., Tian, X., Du, W., Qiu, X., Heese, K., and Wu, M. (2006). Livin promotes Smac/DIABLO degradation by ubiquitin-proteasome pathway. *Cell Death Differ* **13**, 2079-2088.
10. Sun, H., Levenson, J. D., and Hunter, T. (2007). Conserved function of RNF4 family proteins in eukaryotes: targeting a ubiquitin ligase to SUMOylated proteins. *EMBO J* **26**, 4102-4112.
11. Burger, A. M., Gao, Y., Amemiya, Y., Kahn, H. J., Kitching, R., Yang, Y., Sun, P., Narod, S. A., Hanna, W. M., and Seth, A. K. (2005). A novel RING-type ubiquitin ligase breast cancer-associated gene 2 correlates with outcome in invasive breast cancer. *Cancer Res* **65**, 10401-10412.
12. Shih, S. C., Prag, G., Francis, S. A., Sutanto, M. A., Hurley, J. H., and Hicke, L. (2003). A ubiquitin-binding motif required for intramolecular monoubiquitylation, the CUE domain. *EMBO J* **22**, 1273-1281.
13. Raasi, S., and Pickart, C. M. (2003). Rad23 ubiquitin-associated domains (UBA) inhibit 26 S proteasome-catalyzed proteolysis by sequestering lysine 48-linked polyubiquitin chains. *J Biol Chem* **278**, 8951-8959.
14. Kobayashi, S., Sawano, A., Nojima, Y., Shibuya, M., and Maru, Y. (2004). The c-Cbl/CD2AP complex regulates VEGF-induced endocytosis and degradation of Flt-1 (VEGFR-1). *Faseb J* **18**, 929-931.

15. Ali, S., Nouhi, Z., Chughtai, N., and Ali, S. (2003). SHP-2 regulates SOCS-1-mediated Janus kinase-2 ubiquitination/degradation downstream of the prolactin receptor. *J Biol Chem* **278**, 52021-52031.
16. Ahmed, Z., Smith, B. J., and Pillay, T. S. (2000). The APS adapter protein couples the insulin receptor to the phosphorylation of c-Cbl and facilitates ligand-stimulated ubiquitination of the insulin receptor. *FEBS Lett* **475**, 31-34.
17. Matsumoto, M., Hatakeyama, S., Oyamada, K., Oda, Y., Nishimura, T., and Nakayama, K. I. (2005). Large-scale analysis of the human ubiquitin-related proteome. *Proteomics* **5**, 4145-4151.
18. Kirkpatrick, D. S., Weldon, S. F., Tsapralis, G., Liebler, D. C., and Gandolfi, A. J. (2005). Proteomic identification of ubiquitinated proteins from human cells expressing His-tagged ubiquitin. *Proteomics* **5**, 2104-2111.
19. Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006). Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu Rev Cell Dev Biol* **22**, 159-180.
20. Gresko, E., Moller, A., Roscic, A., and Schmitz, M. L. (2005). Covalent modification of human homeodomain interacting protein kinase 2 by SUMO-1 at lysine 25 affects its stability. *Biochem Biophys Res Commun* **329**, 1293-1299.
21. Rodriguez, M. S., Dargemont, C., and Hay, R. T. (2001). SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. *J Biol Chem* **276**, 12654-12659.
22. Li, T., Santockyte, R., Shen, R. F., Tekle, E., Wang, G., Yang, D. C., and Chock, P. B. (2006). A general approach for investigating enzymatic pathways and substrates for ubiquitin-like modifiers. *Arch Biochem Biophys* **453**, 70-74.
23. Norman, J. A., and Shiekhatar, R. (2006). Analysis of Nedd8-associated polypeptides: a model for deciphering the pathway for ubiquitin-like modifications. *Biochemistry* **45**, 3014-3019.
24. Jones, J., Wu, K., Yang, Y., Guerrero, C., Nillegoda, N., Pan, Z. Q., and Huang, L. (2008). A targeted proteomic analysis of the ubiquitin-like modifier nedd8 and associated proteins. *J Proteome Res* **7**, 1274-1287.
25. Sui, G., Affar el, B., Shi, Y., Brignone, C., Wall, N. R., Yin, P., Donohoe, M., Luke, M. P., Calvo, D., Grossman, S. R., and Shi, Y. (2004). Yin Yang 1 is a negative regulator of p53. *Cell* **117**, 859-872.
26. Raymond, F. L. (2006). X linked mental retardation: a clinical guide. *J Med Genet* **43**, 193-200.
27. Lu, Z., Je, H. S., Young, P., Gross, J., Lu, B., and Feng, G. (2007). Regulation of synaptic growth and maturation by a synapse-associated E3 ubiquitin ligase at the neuromuscular junction. *J Cell Biol* **177**, 1077-1089.
28. Hannich, J. T., Lewis, A., Kroetz, M. B., Li, S. J., Heide, H., Emili, A., and Hochstrasser, M. (2005). Defining the SUMO-modified proteome by multiple approaches in *Saccharomyces cerevisiae*. *J Biol Chem* **280**, 4102-4110.
29. Jeon, H. B., Choi, E. S., Yoon, J. H., Hwang, J. H., Chang, J. W., Lee, E. K., Choi, H. W., Park, Z. Y., and Yoo, Y. J. (2007). A proteomics approach to identify the ubiquitinated proteins in mouse heart. *Biochem Biophys Res Commun* **357**, 731-736.
30. Pedrioli, P. G., Raught, B., Zhang, X. D., Rogers, R., Aitchison, J., Matunis, M., and Aebersold, R. (2006). Automated identification of SUMOylation sites using mass spectrometry and SUMOn pattern recognition software. *Nat Methods* **3**, 533-539.

Figure Legends

Figure 1. PTM profiling on protein microarrays. A, Schematic of protein microarray-based profiling of PTM activity. Protein microarrays containing ~8,000 recombinant human proteins spotted onto glass slides in duplicate provide a platform for assessing PTM activity. Reactions are performed 'on-chip' using purified enzymes or extracts prepared from cell or pathological specimens, ATP regenerating system, modifier, and labeled-modifier (e.g. biotin-ubiquitin). PTM conjugation is then detected by incubating the slide with fluorescent-labeled 'binders' (e.g. streptavidin or antibodies) and quantified using a fluorescent slide reader. **B, Profiling substrates of the SCF^{Skp2} ubiquitin ligase.** Purified recombinant SCF^{Skp2} was applied to the protein microarrays in the presence of required co-factors (cyclin A-Cdk2 and Cks1). Insets show ubiquitylation of known SCF^{Skp2} substrate p27^{Kip1} on the protein microarray (red circle) and novel substrates (blue circles). SCF core complex (minus substrate recognition component Skp2) was used as control. **C, Profiling ubiquitin conjugation activity of cellular extracts.** Rabbit reticulocyte lysate or S-100 fractions from HeLa cells were applied to protein microarrays in the presence of ATP regeneration system, ubiquitin, and biotin-labeled ubiquitin. Ubiquitylated substrates (blue circles) and fiducials (positive controls for fluorescence, yellow circles) are highlighted. Similar results were obtained using whole cell extracts from mouse and human cell lines, and human tumor specimens. **D, Multiplex PTM analysis.** Reactions were performed with HeLa cell extract that supported the simultaneous conjugation of ubiquitin, SUMO1, NEDD8, and phosphate to substrates on the protein microarray. The four modifications were then

detected by probing the protein microarrays with antibodies specific for each modification that had been labeled with various fluorescent dyes of non-overlapping excitation and emission wavelengths (see Methods).

Figure 2. *In vivo* confirmation of novel PTM substrates identified on protein microarrays. **A, Ubiquitylation of YY1.** HEK293T cells were transfected with plasmids that express HA-ubiquitin, endogenous YY1 protein then immunoprecipitated from the extracts, and conjugation to ubiquitin determined by Western blot analysis with anti-HA antibodies (left). Immunoprecipitation efficiency was determined by probing blots with anti-YY1 antibodies (right). Immunoprecipitation with IgG antibodies of the same species was used as control. **B, SUMOylation of IGF-1R.** Endogenous IGF-1R was immunoprecipitated from extracts prepared from HEK293T cells and conjugation to SUMO1 determined by Western blot analysis with anti-SUMO1 antibodies. Immunoprecipitation efficiency was determined with anti-IGF-1R antibodies (right). **C, NEDDylation of MUSK and PAK3.** HEK293T cells were transfected with plasmids that express Flag-MUSK or Flag-PAK3 with or without Myc-NEDD8. Extracts were immunoprecipitated with anti-Myc or IgG antibodies of the same species (control) and conjugation to NEDD8 determined by Western blot analysis with anti-Flag antibodies.

Table 1. Ubiquitylated substrates profiled using cell extracts and tumor samples.

BC066929 ¹²	CCDC55 ¹	FGFR3 ²	LOC370014 ¹²³⁵	OR1Q1 ¹	RPL41 ¹	TSPAN17 ¹²³⁴⁵
XM_375359 ¹	CCDC97 ¹	FGFR4 ¹	LOC440295 ¹	PAK1 ⁴	RPS6KA1 ²	TSPO ⁴
ABI1 ¹	CDC2 ¹	FGR ¹²³	LOC51491 ¹	PAK3 ¹	RPS6KA4 ¹⁴⁵	TTK ¹⁴
ABL1 ¹²	CDIPT ¹	FLT1 ¹⁴	LOC51765 ⁴⁵	PBK ¹	RPS6KA5 ¹⁴⁵	TYRO3 ¹⁴
ACBD6 ¹²	CDK2/cyclinA ¹	FLT3 ¹²³⁴	LOC55319 ¹	PDAP1 ¹	RPS6KB1 ⁴	UBADC1 ¹²³⁴⁵
ACVR1B ¹⁴	CDK9/cyclinT1 ¹	FLT4 ¹⁴	LOC645591 ⁴	PDCL ¹³	SCGB1C1 ⁴	UBE2C ¹
AHCYL1 ²	CETN3 ¹²	FRK ¹⁴	LOC83786 ¹	PDGFRA ¹²³⁴	SCYE1 ¹	UBE2E2 ¹⁵
ADRBK2 ¹⁴	CHEK1 ¹	G3BP1 ¹	LOC84714 ⁴	PELI1 ¹	SDCCAG3 ¹	UBE2H ²⁵
AFF4 ¹	CHERP ⁴	GABRA3 ¹	LYN ¹²³	PFDN5 ⁴⁵	SEPT1 ¹	UBE2O ¹²⁴
AIM2 ⁴	CHKA ⁴	GADD45G ¹²	MAGEB1 ¹	PIM1 ¹²⁵	SEPT5 ¹	UBE2S ¹²⁵
AKT1 ²	CHUK ¹	GBA ⁴	MAP2 ¹	PIM2 ¹⁴⁵	SERPINA3 ¹	UBE3A ¹²³⁵
ANKHD1 ¹	CLK3 ⁴	GMNN ¹	MAP2K2 ¹	PKN2 ¹⁴	SGK ⁴⁵	UBQLN2 ²
ANKRD13A ¹²³⁵	CNOT7 ¹²	GNGT1 ⁴	MAP2K3 ¹⁴⁵	PLK1 ¹⁴	SGK3 ⁴	UBXD1 ¹³
ANKRD13D ¹²³⁴⁵	COPE ²	GRK4 ¹⁴⁵	MAP2K6 ¹	PLK3 ¹⁴	SGPL1 ¹	UBXD8 ¹
ANKS4B ¹	COPZ1 ¹⁵	GRK6 ¹⁴	MAP3K2 ¹⁴	POMZP3 ¹	SH3BP5 ¹	VRK3 ¹
APOBEC4 ¹	CSAG1 ¹	GSDMDC1 ¹²	MAP3K9 ¹	PRKCA ¹⁴⁵	SIP1 ⁴	WDFY1 ⁴
ARL6IP4 ¹	CSF1R ¹²³	GSK3B ¹⁴	MAP4K5 ¹⁴⁵	PRKCG ¹	SLAIN2 ³	WDR1 ¹
ASCC2 ¹	CSNK1D ¹⁴	GYG2 ¹	MAPK11 ¹²	PRKCH ¹	SLC6A13 ¹	WEE1 ¹
ASMTL ⁴	CSNK1E ¹	HCK ¹²	MAPKAPK3 ¹	PRKCI ¹	SMCR7 ¹⁵	WIBG ²
ATF6 ¹	CSNK1G1 ¹⁴	HGS ²	MAPKAPK5 ¹²	PRKG2 ¹⁴	SPATS2 ¹	YES1 ¹²³
ATP6V1G1 ¹	CSNK1G3 ⁴	HOMER2 ¹³	MARK2 ¹	PRKX ¹⁴	SPDEF ¹	YY1 ²
ATXN3 ¹²³⁴⁵	CSNK2A1 ¹	HPCAL1 ⁴	MATK ¹	PRRG1 ¹²³⁵	SRMS ⁴	ZAP70 ¹
AURKB ¹	CSNK2A2 ¹⁴	HPGD ¹	MERTK ¹⁴	PSMD4 ¹²³⁴⁵	SRPK1 ¹	ZMYM5 ¹²³
BIN1 ⁴	CUEDC1 ¹²³⁴⁵	IFI44L ⁴	MET ¹	PSRC1 ¹	SRPK2 ¹	ZNF313 ¹
BIRC7 ¹²⁴	CXorf46 ²	IGF1R ¹²³	MINK1 ⁴	PTK2 ¹	SRPK3 ¹	ZNF364 ¹²³⁴⁵
BLK ¹	DAPK1 ⁴	IKBKB ¹	MPG ¹	PTPN5 ¹	STIP1 ¹	ZNF434 ⁴
BMX ¹	DAPK2 ¹	ING5 ¹	MSRB3 ⁴	RAB20 ¹	STK17A ¹⁴	
BRAF ⁴	DHX32 ¹	INSR ¹⁴	MST1R ¹⁴	RABEP2 ²⁵	STK22D ¹	
BTK ¹⁴	DNAJB2 ²	INSRR ¹⁴⁵	MYL5 ¹⁴	RAD23A ¹²³⁴⁵	STK25 ¹	
C10orf97 ¹²³	DNAJC8 ¹⁴	IRAK4 ¹³	MYLK2 ¹⁴	RAF1 ⁴	STK3 ¹⁴⁵	
C11orf52 ¹	DYRK3 ¹⁴	IRF3 ¹	NAP1L2 ¹	RASGRP3 ¹²	STK4 ¹⁴	
C11orf53 ¹	EIF5 ¹	IRS1 ²	NBPF1 ⁴	RASL11B ²	STRAP ¹	
C1orf165 ¹	EPHA1 ¹²³⁴⁵	ITK ¹	NDUFB6 ⁴	RBCK1 ¹	SULF1 ⁴⁵	
C1orf91 ¹	EPHA2 ⁴	JAK2 ¹⁴⁵	NECAP1 ¹	RBM34 ¹	TAOK2 ¹⁴⁵	
C2orf11 ¹	EPHA5 ¹⁴	JAK3 ¹⁴	NECAP2 ¹	RET ¹⁴	TAOK3 ¹⁴⁵	
C2orf13 ⁴⁵	EPHA8 ¹⁴	KDR ¹²³⁴	NEK1 ¹⁴	RHBDD1 ²	TARBP2 ¹	
C9orf78 ¹	EPHB3 ⁴	KIAA1900 ¹	NEK2 ¹	RIOK3 ¹²	TBK1 ¹	
CACNB1 ¹	EPHB4 ¹³	KIF2C ¹	NEK4 ¹	RNF34 ¹³⁴⁵	TCP11 ⁴⁵	
CALCOCO1 ²	ERBB2 ¹²⁵	KIF3B ¹	NEK6 ¹	RNF111 ¹²³⁴⁵	TCP11L1 ¹⁵	
CAMK1 ¹²³	ERBB4 ⁴	KIT ¹	NEK9 ¹⁴⁵	RNF126 ²³⁵	TEC ¹	
CAMK1D ¹	FAM126B ²	LCK ¹	NFKBIB ¹	RNF128 ²	TEK ¹⁴	
CAMK2N1 ¹	FAM112B ¹	LMNA ¹	NGLY1 ²	RNF130 ²	TMEM139 ²	
CAMK2N2 ¹²	FAM50A ¹	LOC10572 ²	NMT1 ¹	RNF185 ¹²³⁵	TNIK ¹	
CAMKIIalpha ¹	FES ¹	LOC112860 ⁴	NR4A1 ¹	RNF4 ¹²⁴⁵	TNIP2 ¹²⁵	
CAMKIIdelta ¹	FER ⁴	LOC115460 ¹	NTRK1 ¹	ROR1 ¹	TOM1 ¹²⁵	
CASQ2 ¹	FGF21 ²	LOC120376 ¹	NTRK2 ¹	ROR2 ⁴⁵	TOM1L2 ¹²³⁴⁵	
CAT ¹	FGFR1 ¹²	LOC121457 ⁴	NTRK3 ¹	ROS1 ¹	TRIM44 ¹	
CCDC12 ¹	FGFR2 ¹	LOC284440 ⁴	NUAK1 ¹	RPAIN ¹²	TRIM52 ¹²³⁴⁵	

¹Rabbit reticulocyte lysates, ²Mouse embryonic fibroblasts, ³Human foreskin fibroblasts, ⁴HeLa cell S-100 fractions, ⁵Breast tumor specimens.

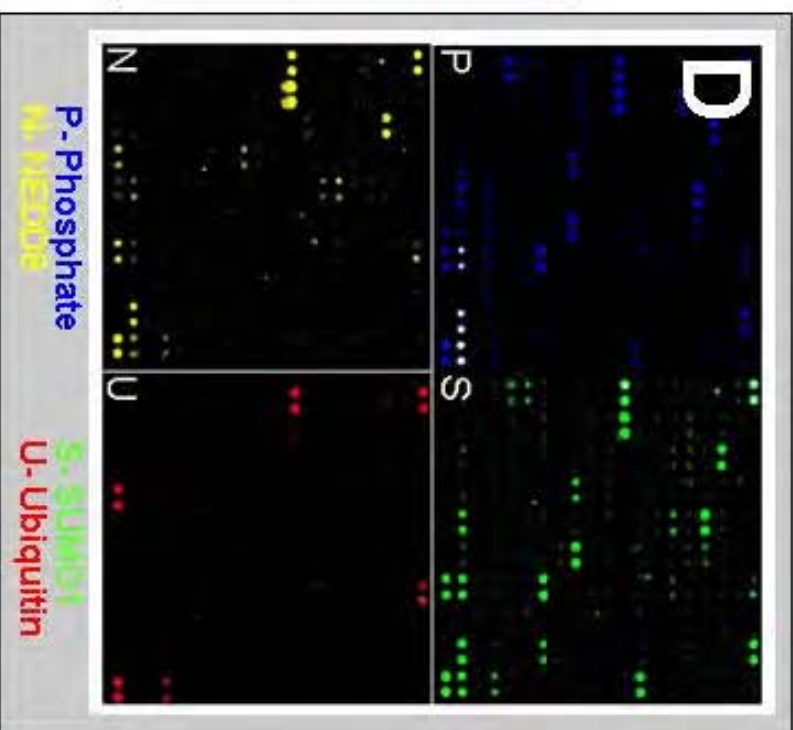
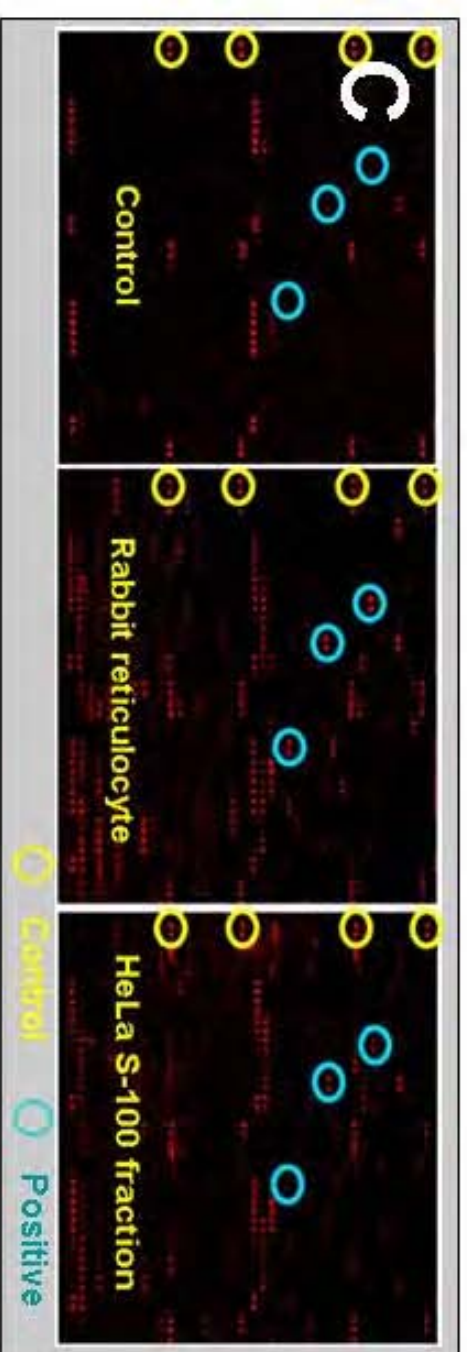
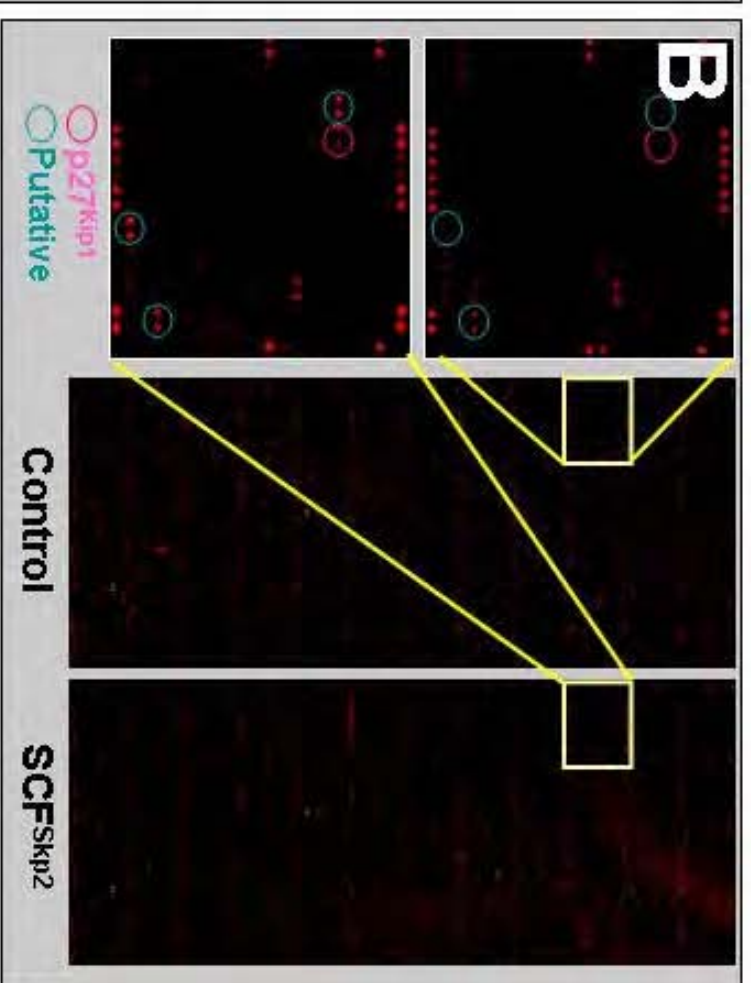
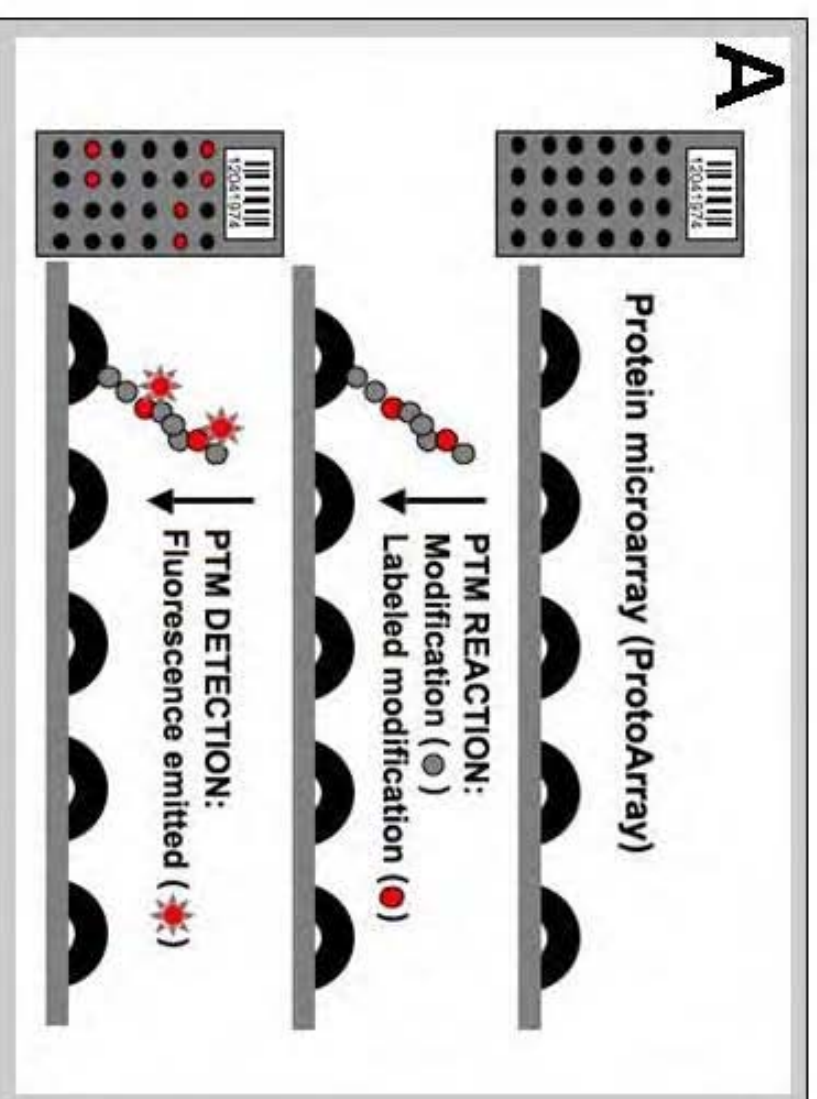
TABLE 2. Ubiquitin, NEDD8, or SUMO1 conjugated proteins identified on protein microarrays.

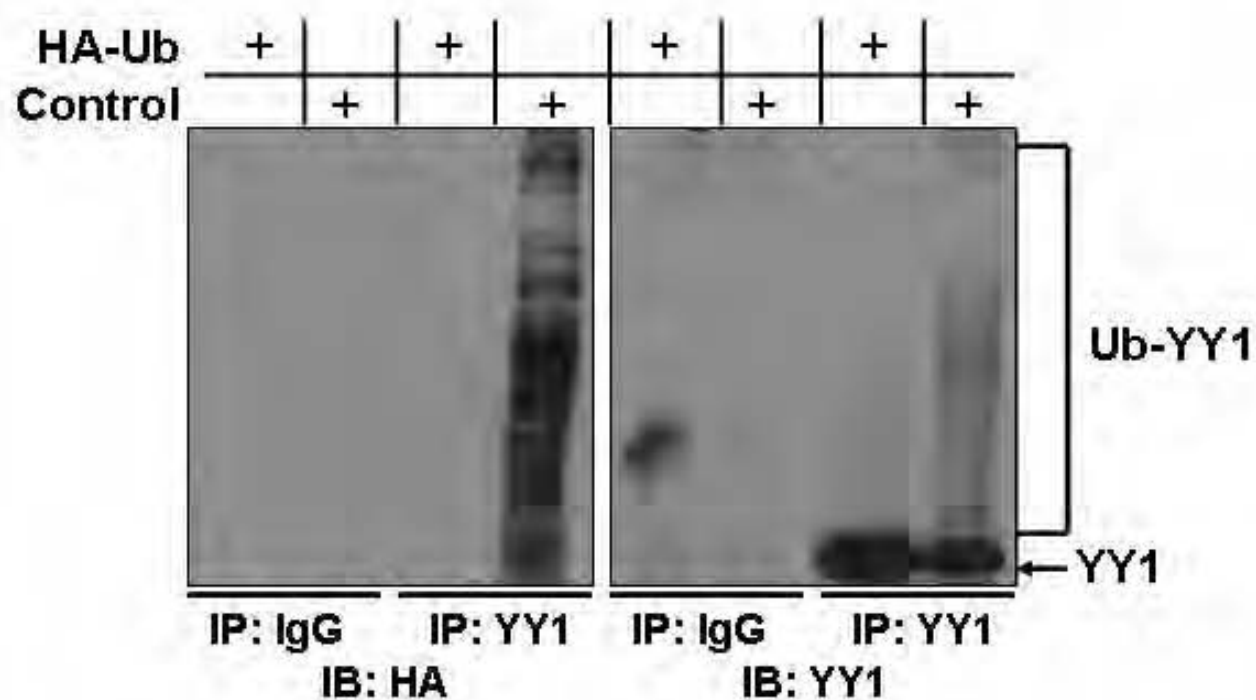
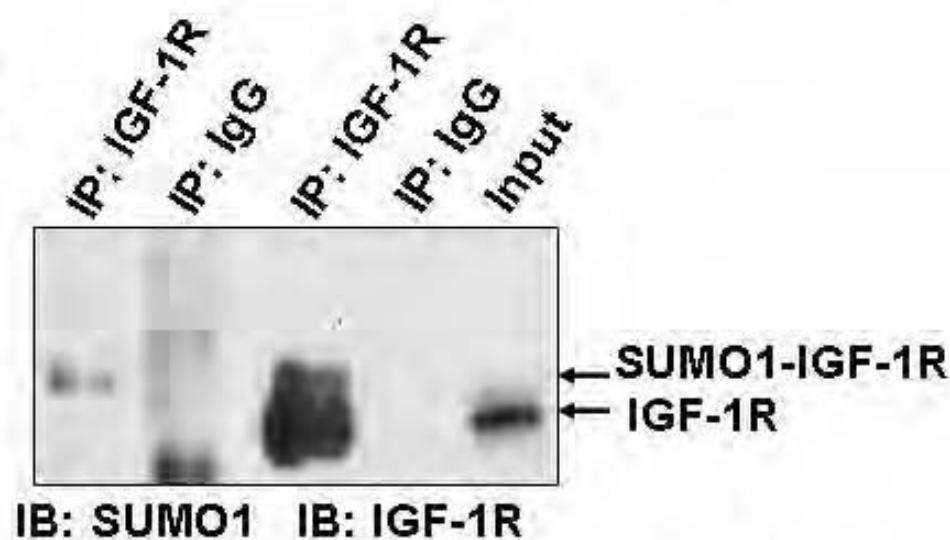
UBIQUITYLATION				NEDDYLATION		SUMOYLATION
UPS-Associated		UBIQUITIN SUBSTRATES		NEDD8 SUBSTRATES		SUMO1 SUBSTRATES
ACVR1B*	MST1R*	ADRBK2	MYLK2	ANKHD1	LSM3	<i>ADRBK1</i>
ATXN3	PDGFRa*	ANKRD13D	NEK1	ANKRD13D	MAP3K10	<i>AKT2</i>
BTK*	PLK1*	CSNK1D	NEK9	ANKRD17	MAP3K11	<i>CDK5</i>
CAT*	PLK3*	CSNK1G1	PIM2	ANKRD39	MAP3K9	<i>CENPB</i>
CUEDC1	PRKCa*	CSNK2A2	PKN2	ANKS4B	MATK	<i>COPE</i>
FLT1*	PRKCg*	DYRK3	PRKX	BTK	MCC	<i>FES</i>
FLT3*	PSMD4	EPHA1	ROS1	CCDC69	MINK1	<i>FGFR3</i>
GSK3-beta*	RAD23A	EPHA5	RPS6KA4	CENPB	MST1R	<i>FGR</i>
INSR*	RET*	FLT4	RPS6KA5	CETN3	NAP1L1	<i>FYN</i>
ITK*	<u>RNF4</u>	FRK	STK3	CHEK1	NFKBIB	<i>HIPK3</i>
JAK2*	<u>RNF111</u>	GRK4	STK4	CSNK2A1	OTUD6B	<i>HK1</i>
JAK3*	TTK*	GRK6	STK17A	CUEDC1	PAIP2	<i>ING3</i>
<u>LIVIN</u>	UBADC1	INSRR	TAOK2	CXorf48	PAK1	<i>JAK3</i>
MAP3K2*	UBE2O	KIAA1900	TAOK3	DIXDC1	PAK3	<i>LCK</i>
MAP4K5*	ZNF364	MAP2K3	TEK	EIF2B2	PBK	<i>LENG4</i>
		MCAK	TRIM52	EPHA1	PDCL	<i>MAPKAPK5</i>
		MERTK	TSPAN17	EPHB4	PEX19	<i>MERTK</i>
		MYL5	TYRO3	FAIM	PIM1	<i>PAK3</i>
				FGR	PRKCA	<i>PBK</i>
				GCC1	PRKCE	<i>RBCK1</i>
				GOPC	PSCD1	<i>RIPK2</i>
				GSDMDC1	RAD23A	<i>RNF4</i>
				LCK	RGS20	<i>RPS6KA3</i>
				LGALS3	RPS6KB1	<i>STK3</i>
				LMNA	TOM1L2	<i>VPS29</i>
				LOC126382	TRIM44	<i>ZMYM5</i>
				LOC57596	UBOX5	

Substrates shown for ubiquitin are common to both rabbit reticulocyte lysate and HeLa S-100 fractions. Underlined, E3-associated; *, known substrate of ubiquitylation; **Bold**, high homology to proteins known to be ubiquitylated; **Highlighted**, substrates also common to human fibroblasts; *Italics*, SUMO1 substrates containing SUMO consensus sequences (yKxE/D). UPS, ubiquitin proteasome system.

Table 3. Changes in protein ubiquitylation associated with human tumor progression.

INCREASED	PROTEIN FUNCTION	DECREASED	PROTEIN FUNCTION
RAD23A	Involved in post-replication repair of UV-damaged DNA	ATXN3	Machado-Joseph disease gene product, nucleotide excision repair
MARK2	Microtubule binding protein, Ser/Thr kinase	FLT4	VEGFR3, angiogenesis, RTK
TRIM52	E3 ligase activity	SULF1	Inhibits tumor growth
ZNF364	BCA E3 ligase	PIM2	Oncogene, Ser/Thr kinase
TSPAN17	FBXO23, cell adhesion	PRRG1	SH3, binds ubiquitin
DDR2	RTK, metastasis	NEK9	Maintains proper G ₁ and S progression
MYLK2	Cytoskeletal regulation, cell motility, Ser/Thr kinase	FGF21	Growth factor activity
ALK	Anaplastic lymphoma kinase, RTK, tumor aggressiveness	SLAIN2	Unclassified
TFPI2	Tissue factor pathway inhibitor 2	CAMKK2	Calcium signaling
BC050434	Unknown	PLK3	Mitosis, DNA damage checkpoint activation



A**B****C**